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The octadecaneuropeptide [diazepam-binding inhibitor (33–50)] exerts potent anorexigenic effects in rodents

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Abstract

The effects of intracerebroventricular administration of the octadecaneuropeptide ODN on food intake have been investigated in rat and mouse. In rats deprived of food from 9:00 a.m. to 7:00 p.m., i.c.v. injection of ODN (30 to 100 ng) provoked a dose-dependent reduction of food consumption during the following 12-h nocturnal period. At a dose of 100 ng, ODN almost completely suppressed food intake. Treatment of rats with diazepam (2 mg/kg s.c.; 15 min before ODN administration) did not affect the anorexigenic response evoked by 100 ng ODN. Continuous i.c.v. infusion of ODN (10 ng/h during 15 days) using osmotic minipumps, significantly reduced food intake during the 2nd, 3rd and 4th days of treatment. The decrease in food consumption was associated with a significant reduction in body weight, which persisted during the 15-day duration of the experiment. In mice deprived of food for 18 h, i.c.v. administration of a low dose of ODN (5 ng) significantly reduced food intake. Treatment of mice with diazepam (1 mg/kg s.c.; 10 min before ODN administration) did not prevent the inhibitory effect of ODN (100 ng) on food intake. The C-terminal octapeptide fragment of ODN mimicked the anorexigenic effect of the intact peptide. Taken together, the present data demonstrate that i.c.v. injection of ODN causes, in both rat and mouse, a long-lasting anorexigenic effect that is not mediated through central-type benzodiazepine receptors. The biologically active region of ODN appears to be located in the C-terminal domain of the peptide.

Keywords: Diazepam-binding inhibitor fragment; Endozepine; (Rat); (Mouse); Food intake

1. Introduction

Diazepam-binding inhibitor (DBI) is an 86 amino-acid polypeptide that has been initially isolated from the rat brain on the basis of its ability to displace diazepam from its binding sites (Guidotti et al., 1983). Proteolytic cleavage of DBI generates several biologically active fragments, including the triakonta-tetraneuropeptide=diazepam-binding inhibitor-(17–50) (TTN) (Slobodyansky et al., 1989) and the octadecaneuropeptide=diazepam-binding inhibitor-(33–50) (ODN) (Ferrero et al., 1984), which are all designated by the generic term endozepines (Tonon et al., 1994). Pharmacological studies have shown that ODN interacts predominantly with central-type benzodiazepine receptors (Ferrero et al., 1986; Slobodyansky et al., 1989; Berkovich et al., 1990) while triakonta-tetraneuropeptide is a selective ligand for peripheral-type (i.e. mitochondrial) benzodiazepine receptors (Slobodyansky et al., 1989; Berkovich et al., 1990). Endozepines may also activate a membrane receptor positively coupled to phospholipase C through a pertussis toxin-sensitive G-protein (Patte et al., 1995; Gandolfo et al., 1997).

Endozepines are widely distributed in the central nervous system (Alho et al., 1989; Tonon et al., 1990; Costa and Guidotti, 1991; Malagon et al., 1993). In particular, high concentrations of endozepines have been found in the dorso-medial and the ventro-medial nuclei as well as in the lateral area of the hypothalamus (Alho et al., 1985; Malagon et al., 1993), which play a major role in the

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control of food intake. It has also been shown that agonists of central-type benzodiazepine receptors produce hyperphagic responses in various species (Cooper, 1980, 1989). For example, central-type benzodiazepine receptors agonists induce a substantial increase in food consumption in rats trained to eat a palatable diet (Cooper et al., 1985) and this hyperphagic response is blocked by the specific central-type benzodiazepine receptors antagonist flumazenil (Ro 15-1788) (Cooper et al., 1985; Cooper and Moores, 1985). Although many peptides are known to modulate feeding behavior (for review see Schwartz et al., 2000), the effect of endozepines on food intake has never been investigated.

In the present study we have examined the effect of intracerebroventricular (i.c.v.) administration of ODN and its C-terminal octapeptide fragment on food consumption in rat and mouse.

2. Materials and methods

2.1. Animals

Experiments were carried out on male Sprague Dawley rats (250–280 g) and male Swiss albino mice CD1 (22–25 g) purchased from Charles River (Saint-Aubin lès Elbeuf, France). Rats and mice were housed by 4 and 30, respectively, in Makrolon cages ($l = 38$ cm, $w = 24$ cm, $h = 18$ cm), with free access to water and food (cylindrical pellets, diameter 12 mm, weighing 4–5 g, Ref A04, UAR, Villemoisson-sur-Orge, France). The animals were kept in a ventilated room at a temperature of 21 ± 1°C, under a 12-h light/12-h dark cycle (light on between 7:00 a.m. and 7:00 p.m.).

Animal manipulations were performed according to the recommendations of the French Ethical Committee and under the supervision of authorized investigators.

2.2. Acute intracerebroventricular injections

Rats were anaesthetized with chloral hydrate (400 mg/kg, i.p.) and a small area of the skin (about 25 mm^2) was dissected out. An incomplete drilling of the skull was made with a dentary miller, 2 mm posterior and 1.8 mm lateral to bregma, according to Albe-Fessard et al. (1966). An organo-mercurial antiseptic was applied for preventing infection. Forty eight hours later, an i.c.v. injection (20 μl) was made free-hand in the left ventricle, in about 5 s, with a microsyringe (Hamilton 50 μl) connected to a needle (diameter 0.5 mm), of which the median part of the bevel protruded 5 mm from a guard limiting its penetration into the brain. I.c.v. injections were performed by an experienced investigator. Pilot experiments that consisted in injecting a dye solution (diluted India ink) had shown, after brain sectioning, that the injection was successfully performed in the left ventricle in more than 95% of the trials.

In mice, i.c.v. injections (10 μl) were made in the left ventricle, in about 3 s, with a microsyringe (Hamilton, 50 μl) connected to a needle (diameter 0.5 mm), of which the median part of the bevel protruded 3.5 mm from a guard, limiting its penetration into the brain of manually immobilized mice, according to the procedure of Haley and McCormick (1957).

2.3. Semi-chronic intracerebroventricular administration

Rats were anaesthetized with chloral hydrate (400 mg/kg, i.p.) and an incomplete drilling of the skull was made as described above. A 5-mm long stainless steel needle (Brain infusion kit, Alzet, Charles River, France) was implanted in the lateral ventricle and the cannula was connected by a catheter to an Alzet osmotic minipump (model 2002), inserted subcutaneously (s.c.) in the neck region. The pump was filled with either ODN or saline and was incubated in sterile saline at 37°C for 4 h prior to the implantation. This procedure ensured the immediate infusion at a regular flow (10 ng/0.5 μl/h) after s.c. implantation for a 15-day period. The correct location of the cannula was verified at the end of the infusion by an injection of methylene blue.

2.4. Food consumption measurements

In rats deprived of food for 10 h (9:00 a.m. to 7:00 p.m.), food intake was measured with a feeding monitor apparatus (Columbus Instruments, OH, USA). This device was modified from its original conception in order to prevent: (i) the storing up behavior of the rat, which would remove the pellets from the pan of the balance; (ii) the leaning of animal with forepaws on the pan; and (iii) the emission of dejections on the pan. Food intake was determined at selected intervals by a computer.

Mice deprived of food for 18 h (6:00 p.m. to 12:00 a.m.) were placed in individual cages ($l = 24$ cm, $w = 10.5$ cm, $h = 6.5$ cm), with access to a previously weighed pellet of food. At various times, the pellet was briefly (< 20 s) removed with plastic pliers and weighed.

2.5. Drugs

Rat/mouse ODN (Gln-Ala-Thr-Val-Gly-Asp-Val-Asn-Thr-Asp-Arg-Pro-Gly-Leu-Leu-Asp-Leu-Lys) and its C-terminal octapeptide fragment (Arg-Pro-Gly-Leu-Leu-Asp-Leu-Lys) were synthesized by solid phase methodology, as previously described (Leprince et al., 1998). The peptides were dissolved in saline, using plastic tubes, just before i.c.v. injection or osmotic minipump loading. Diazepam (injectable solution of Valium®; Roche, Neuilly-sur-Seine,

France) was diluted in saline and administered s.c. at a dose of 2 mg/kg (rat) or 1 mg/kg (mouse).

2.6. Statistics

The data are expressed as means ± S.E.M. The Student's *t*-test was used for comparison of the mean values between two groups; analysis of variance (ANOVA) one-way was used for comparison between two groups. ANOVA two-way was used for comparison between multiple groups.

3. Results

Acute i.c.v. administration of ODN to rats deprived of food for 10 h (from 9:00 a.m. to 7:00 p.m.) produced a dose-dependent inhibition of food intake during the nocturnal period (Fig. 1). A significant reduction of food consumption was already observed at the lowest dose of ODN tested (30 ng/rat). At a 30-ng dose, ODN injection provoked a 46% reduction in food intake during the entire nocturnal period ($P < 0.01$). At the highest dose tested (100 ng/rat), ODN almost completely suppressed feeding (Fig. 1).

Acute administration of ODN (100 ng) to rats deprived of food for 18 h reduced by about 75% food intake during the following 2 h (Fig. 2). In control rats, treatment with diazepam (2 mg/kg, s.c.) 15 min before i.c.v. saline injection provoked a significant reduction ($P < 0.01$) of food consumption during the first hour. The inhibitory effect of ODN on food consumption was not affected by diazepam treatment (Fig. 2).

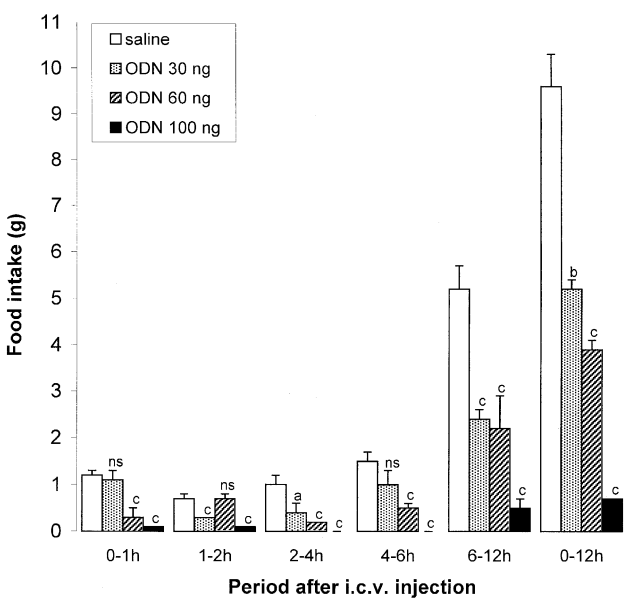

Fig. 1. Time-course of the effect of ODN on nocturnal food intake in rats deprived of food during 10 h. Rats were placed at 9:00 a.m. in individual test cages, without food and with water ad libitum. At 7:00 p.m. the animals were injected i.c.v. (20 μl) with saline or ODN (30, 60 or 100 ng) and immediately placed again in their individual test cages with food available. Food intake was determined during the periods indicated. Mean ± S.E.M. of 12 rats per group. One-way ANOVA; [a] $P < 0.05$, [b] $P < 0.01$, [c] $P < 0.001$, ns: not statistically significant, as compared to saline-injected controls.

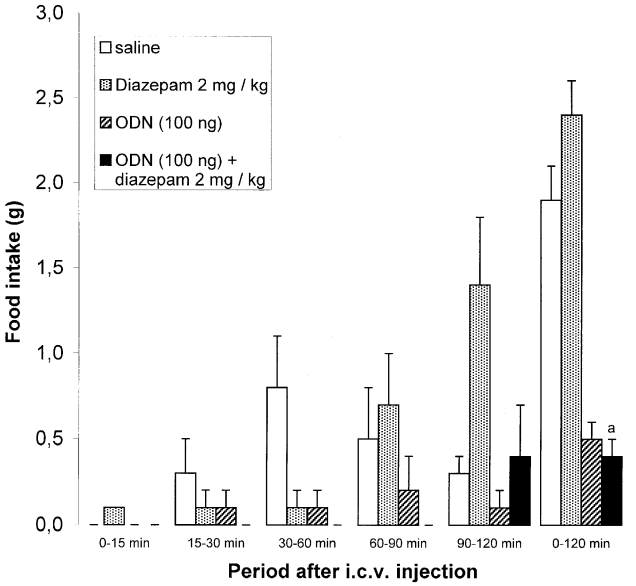

Fig. 2. Effect of diazepam on the ODN-induced inhibition of food intake in rats deprived of food during 18 h. Rats were placed at 6:00 p.m. in individual test cages, without food and with water ad libitum. The next day, at 12:00 a.m., diazepam (2 mg/kg) was administered s.c. 15 min before i.c.v. injection of either 20 μl saline or ODN (100 ng) and food was immediately available. Food intake was determined during the periods indicated. Mean ± S.E.M. of six rats per group. [a] Not significantly different as compared to the ODN-treated group for the 0–120 min period (two-way ANOVA followed by Fisher's least significant difference test).

The effect of semi-chronic (15 days) i.c.v. administration of ODN on food consumption and body weight was tested by using osmotic minipumps (Fig. 3). In control rats implanted with minipumps containing only the saline vehicle, a marked reduction in food intake was observed during the 6-day period following surgery. Thereafter, food consumption stabilized at about 25 g/day. Continuous infusion of ODN (10 ng/h) significantly reduced food intake during the 2nd, 3rd and 4th days of testing (Fig. 3A). Although daily food intake during the 5th, 6th, 7th, 8th, and 9th days did not differ significantly from that of controls, the whole amount of food eaten during this 5-day period was significantly ($P < 0.05$) lower in ODN-treated rats (95 ± 9 g) than in controls (119 ± 5 g). Finally, no rebound in food intake was observed throughout the 15-day period (Fig. 3A).

Continuous i.c.v. infusion of ODN produced a significant decrease in the body weight of rat throughout the 15-day period (Fig. 3B). A significant effect of ODN was already observed after 2 days of treatment and the maximum reduction in body weight occurred after 7 days.

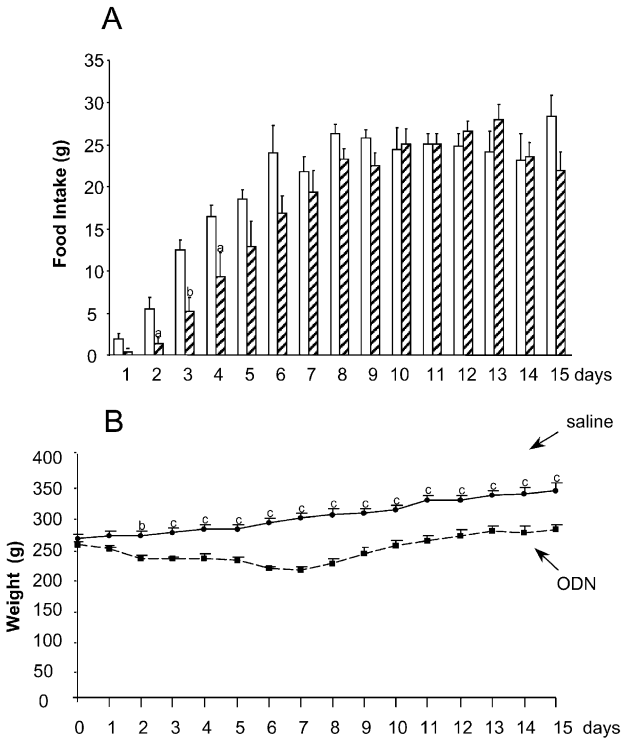

Fig. 3. Effect of continuous i.c.v. infusion of ODN in rat on food intake (A) and body weight (B). The animals were implanted with osmotic minipumps that delivered i.c.v. (0.5 μl/h) saline (□) or ODN (10 ng/h; ▨). Daily food intake (upper panel) and body weight (lower panel) were measured each day at 9:00 a.m. during 15 days after implantation. Means ± S.E.M. of seven to eight rats per group. Student's *t*-test; $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$ as compared to saline controls.

Thereafter, the weight of the ODN-treated rats remained about 20% lower than that of the control animals (Fig. 3B).

In mice deprived of food for 18 h, acute i.c.v. administration of ODN provoked a significant reduction of food intake during the following 2 h (Table 1). The lowest dose of ODN tested (5 ng/mouse) elicited a significant effect only during the first hour after injection. At higher doses (10 and 100 ng/mouse), a significant decrease in food consumption was observed during 2 h (Table 1). I.c.v. administration of the octapeptide fragment corresponding to the C-terminal sequence of ODN mimicked the anorexigenic effect of ODN during the second hour after injection (Table 1).

In order to determine the delay of the anorexigenic effect of ODN and its octapeptide fragment, we have investigated the influence of the lag period between i.c.v. injection of each peptide (100 ng/mouse) and the time of food presentation. As shown in Fig. 4, both peptides reduced feeding when food was presented immediately after the injection and were still effective when food was presented 2 h later.

In mice deprived of food for 18 h, treatment with diazepam (1 mg/kg, s.c.) reduced food intake during the first 20-min test period following food presentation. Thereafter, a rebound in food consumption was observed in diazepam-treated mice during the following 40-min period (Fig. 5). I.c.v. injection of ODN (100 ng/mouse) significantly reduced food intake during the 4-h test period.

Table 1
Time-course of the effect of ODN or its C-terminal octapeptide fragment on food intake in mice deprived of food during 18 h

Period after i.c.v. injection	Food intake (mg) in mice injected with:			
	Saline	ODN, 5 ng	ODN, 10 ng	ODN, 100 ng
0–1 h	537 ± 35	401 ± 22[b]	405 ± 42[a]	387 ± 40[b]
1–2 h	153 ± 20	112 ± 21 ns	96 ± 17[a]	75 ± 18[b]
2–4 h	194 ± 27	148 ± 32 ns	147 ± 36 ns	159 ± 32 ns
	Saline		Octa, 10 ng	Octa, 100 ng
0–1 h	537 ± 35		507 ± 53 ns	431 ± 48 ns
1–2 h	153 ± 20		89 ± 19[a]	90 ± 23[a]
2–3 h	194 ± 27		184 ± 38 ns	167 ± 50 ns

Mice were placed at 6:00 p.m. in individual cages without food and with water ad libitum. The next day, at 12:00 a.m., the animals were injected i.c.v. (10 μl) with saline or ODN (5, 10 or 100 ng) or octapeptide(octa; 10 or 100 ng) and immediately placed again in their individual test cages with food available. Mean ± S.E.M. of 20 mice per group. ANOVA one-way, $^{a}P < 0.05$, $^{b}P < 0.01$, ns: not statistically significant, as compared to saline-injected controls.

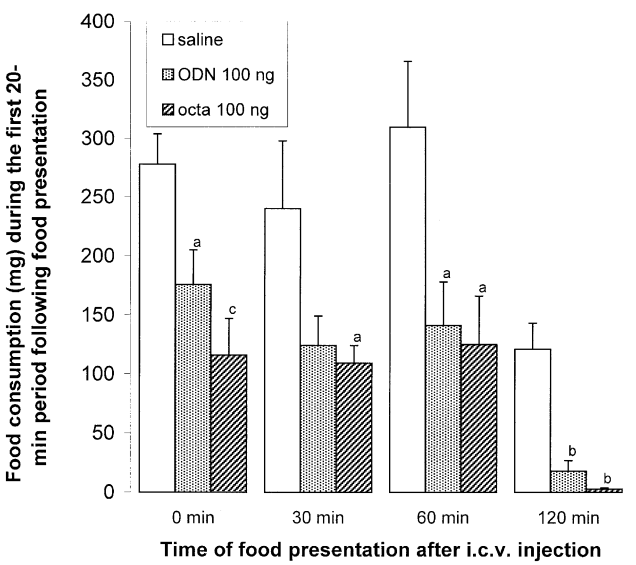

Fig. 4. Influence of the time lag between i.c.v. administration of ODN or its C-terminal octapeptide and food presentation on the anorexigenic effect of the two peptides in mice deprived of food during 18 h. Mice were placed at 6:00 p.m. in individual test cages, without food and with water ad libitum. The next day, at 12:00 a.m., the animals were injected i.c.v. (10 μl) with saline or ODN (100 ng) or its C-terminal octapeptide fragment (octa; 100 ng) and placed in the test cage where food was presented immediately or after 30, 60 or 120 min. Food intake was determined during the 20-min period following food presentation. Mean ± S.E.M. of 10 mice per group. Student's *t*-test; $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$, ns: not statistically significant, as compared to saline-injected controls.

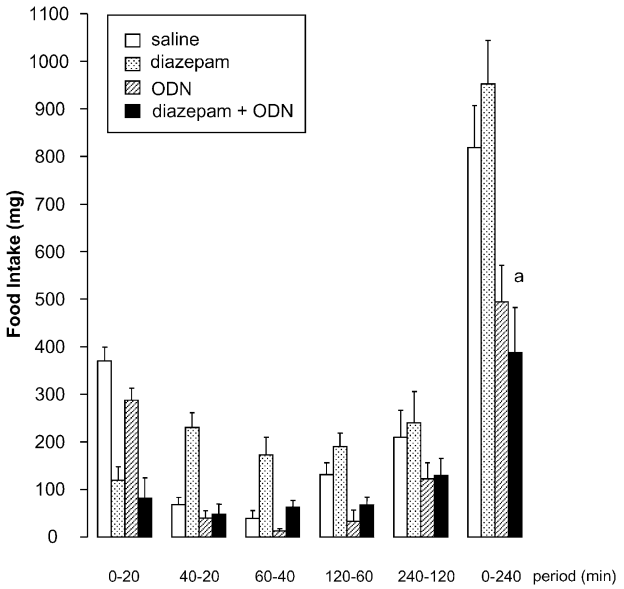

Fig. 5. Effect of diazepam on the ODN-induced inhibition of food intake in mice. Diazepam (1 mg / kg) was administered s.c. 20 min before i.c.v. injection of either 10 μl saline or ODN (100 ng). 10 min after the latter injection, the animals had access to food pellets. Food intake was determined during the periods indicated. Mean ± S.E.M. of 10 mice per group. [a]Not significantly different as compared to the ODN-treated group for the 0–240 min period (two-way ANOVA followed by Fisher's least significant difference test).

Diazepam did not affect the anorexigenic response induced by ODN (Fig. 5).

4. Discussion

The present study has demonstrated that i.c.v. administration of low doses of ODN to food-deprived rats and mice causes a substantial reduction in food consumption. To our knowledge, these data provide the first evidence for an anorexigenic effect of peptides of the endozepine family.

A significant inhibitory effect of ODN on food consumption was already observed after administration of doses of the peptide as low as 30 ng/ rat or 5 ng/mouse. These doses are 3 to 100 times lower than the minimum effective doses described for other anorexigenic peptides including cholecystokinin (Kadar et al., 1985), α-melanocyte-stimulating hormone (Poggioli et al., 1986; Abbott et al., 2000) or pituitary adenylate cyclase-activating polypeptide (Morley et al., 1992; Chance et al., 1995; Mizuno et al., 1998). It thus appears that ODN is one of the most potent anorexigenic peptides reported so far.

It has been previously shown that intraduodenal infusion of ODN provokes cholecystokinin release from intestinal endocrine cells (Li et al., 2000). Since cholecystokinin acts at the periphery as a satiety hormone (Ridelberger, 1994), this observation raised the question as to whether ODN, injected centrally, could cross the blood–brain barrier and act as an endocrine factor to reduce food consumption. This hypothesis was very unlikely inasmuch as the effective dose of ODN injected i.c.v. was apparently too low to produce, after diffusion to the periphery, a significant concentration in the systemic circulation. In fact, we found that intravenous administration of 1000 ng of ODN in mice (i.e. 200-fold the i.c.v. effective dose) did not affect food intake, demonstrating that ODN exerts its anorexigenic effect centrally.

Previous studies have shown that the C-terminal octapeptide fragment of ODN mimics some of the behavioral actions of ODN such as the anxiogenic effect (Garcia de Mateos-Verchere et al., 1998a), the inhibitory effect of ODN on apomorphine-induced yawning (Garcia de Mateos-Verchere et al., 1998b) and pentylenetetrazol-induced convulsions (Garcia de Mateos-Verchere et al., 1999). It has also been found that the behavioral effects provoked by the octapeptide were more precocious than those evoked by ODN, suggesting that ODN may act as a prodrug capable of generating a biologically active fragment (Garcia de Mateos-Verchere et al., 1998a,b, 1999). The present report shows that, although the C-terminal octapeptide also provoked a significant inhibition of food intake, the time lag between the injection of the peptide and the response was not shorter, indicating that the anorexigenic effect of ODN cannot be accounted for by the formation of a proteolytic fragment.

In rat, the anorexigenic effect of ODN was observed whether the animals had been deprived of food only during the diurnal phase preceding the test or during 18 h, i.e. during the nocturnal phase corresponding to their main period of feeding and the following diurnal phase. In both rat and mouse, diazepam alone produced a biphasic effect on feeding behavior: diazepam first caused a transient inhibition of food intake that could be ascribed to its sedative effect; then, diazepam increased food consumption. During this feeding-stimulating phase induced by diazepam, ODN still retained its inhibitory effect on food intake, indicating that the anorexigenic action of ODN is not mediated through central-type benzodiazepine receptors. Although ODN has been characterized as a selective inverse agonist of central-type benzodiazepine receptors (Guidotti et al., 1983; Ferrero et al, 1984, 1986), several effects of ODN cannot be accounted for by an action on the $GABA_A$ benzodiazepine receptor complex (Gandolfo et al., 1997). In particular, it has been shown that the effect of ODN on calcium mobilization in rat astrocytes is mediated through activation of a metabotropic receptor positively coupled to phospholipase C (Patte et al., 1995; Lamacz et al., 1996). Whether the anorexigenic effect of ODN involves this latter receptor type remains to be determined.

Semi-chronic central infusion of ODN significantly inhibited food intake during the first 4 days of treatment, indicating that the ODN-induced anorexia does not give

rise to rapid tolerance. Although the anorexigenic effect of ODN vanished after 7 days, a significant loss in body weight was observed throughout the 15-day treatment and no rebound in food consumption occurred. The lack of effect of ODN during the second week of treatment may be ascribed to a desensitization process. Alternatively, the perfusion rate or the peptide stability might have been altered during the semi-chronic infusion.

Anxiety is known to reduce food intake. For instance, a starved rat introduced in a new environment, with food available, does not eat before it gets familiar with its environment (Soubrié et al., 1978) and this delay is shortened by anxiolytic agents. Several reports have shown that ODN induces anxiety in rodents (Bender and Hertz, 1986; Garcia de Mateos-Verchere et al., 1998a), suggesting that the anorexigenic effect of the peptide might be ascribed to its anxiogenic properties. Three observations however indicate that this is not the case. (i) The present study has shown that the action of ODN on food intake is not mediated through central-type benzodiazepine receptors while the anxiogenic effect of the peptide can be accounted for by its inverse agonistic activity on central-type benzodiazepine receptors. (ii) In some of our experiments, rats were accustomed to the test cage during the diurnal period and this habituation did not impair the anorexigenic effect of ODN. Similarly, in the semi-chronic experiments, reduction of food intake was observed although the animals were totally familiar with their environment. (iii) In mice, the time lag between the introduction of the animals into the test cage (i.e. immediately after ODN injection) and food presentation could be increased up to 2 h without alteration of the anorexigenic action of the peptide. Altogether, these data clearly indicate that the anorexigenic effect of ODN is not attributable to its anxiogenic activity.

In addition, we have not observed modifications of locomotor activity suggesting either a sedation or on the contrary a psychostimulant activity; there was no more stereotyped behaviours or seizures that could have aspecifically interfered with the feeding behaviour.

In conclusion, central (but not peripheral) administration of low doses of ODN markedly inhibits food intake in both food-deprived and normally fed rodents. The anorexigenic effect of ODN is long lasting, does not give rise to rapid tolerance and is associated with substantial weight loss. The action of ODN is not mediated through central-type benzodiazepine receptors, suggesting that it may be possible to develop ODN analogues that would selectively inhibit food consumption without producing anxiogenic effect.

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